ISOLATION AND ASSAY OF THE NEMATOCYST TOXIN OF METRIDIUM SENILE FIMBRIATUM

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The study of the immune mechanisms of marine invertebrates is inhibited by our lack of knowledge of the infectious diseases of these organisms. However, the many commensal relationships which exist between a variety of organisms and members of the Phylum Coelenterata suggest that a study of induced immunity to nematocyst toxins would at least yield information pertinent to the development of an understanding of antitoxic immunity in marine invertebrates.

There have been a number of attempts at the purification and description of these toxins (Cosmovici, 1925; Cantacuzène, 1926; Cantacuzène and Damboviceanu, 1934a, 1934b; Richet and Portier, 1936; Sonderhoff, 1936; Welsh, 1955). In all cases the material isolated represents extracts of the whole animal or some of its organs, *e.g.*, tentacles or acontia. As has been pointed out by Hyman (1940) in no case can it be certain that the material isolated is actually from nematocysts, the stinging capsules, and is not some toxic tissue component which normally does not play a role in the defensive or food gathering activities of the animal.

These present studies were carried out to develop a method of obtaining purified suspensions of nematocysts from sea anemones (*Actiniaria*) in order to obtain a toxic preparation which could be considered to be nematocystic in origin and could be used in studies on the antitoxic response of a variety of marine invertebrates.

Isolation of Nematocysts

The entire anemone was used as a source of nematocysts. Attempts to isolate nematocysts by enzymatic digestion of the surrounding tissues with pepsin, trypsin, ficin, and papain always resulted in damage to these structures, but the physical methods of separation described below have yielded suitable material. A number of variations in the method of preparation have been used, and the properties of the resulting materials have varied with the method. A few of these variations are included here since they illustrate species differences with regard to ease of purification and nature of the nematocysts, and these variations in method may be of help in similar investigations on other members of the phylum.

The anemones were first cleaned of adherent debris by placing them for a few days in a coarse wire mesh basket in an aquarium with running sea water. Approximately 500 grams, wet weight, of the animals were macerated in a Waring Blendor with 500 ml. of 1 *M* sucrose in sea water. An additional 500 ml. of this suspending medium was added, and the material was passed through a series of graded screens with openings of 1.168, 0.589, 0.295, and 0.147 mm. with the aid

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of suction. Tyler Standard Screens fastened to a Buchner funnel with masking tape were used. These screens remove the large particles of tissue from the suspension and allow the nematocysts to pass through, along with fine tissue debris, dissolved tissue components, and very fine sand. Filtrates from anemones, whose tissues contain symbiotic algae, bear these organisms as an additional contaminant. Upon centrifugation at 1000 rpm. for 15 minutes, the nematocysts were collected along with the sand, fine tissue debris, and algal cells if these were present. The sediment was washed free of dissolved tissue constituents by repeated re-suspension in the sucrose solution and re-centrifugation. This procedure also removed a considerable amount of the fine tissue debris. The nematocysts were purified further by differential centrifugations of 15 minutes and 15 seconds at top speed in a small International Clinical Centrifuge with a bucket head. The longer centrifugation left most of the fine tissue debris in suspension while the nematocysts were collected in the sediment. The shorter centrifugation left the majority of nematocysts in suspense but removed the sand. Five or six pairs of centrifugations were usually sufficient.

Three criteria for the success of any method were employed, *i.e.*, purity of the suspensions, susceptibility of resulting nematocysts to artificial discharge, and toxicity of the material released on discharge. Particularly good results were obtained in the case of *Metridium senile fimbriatum*. Characteristics of these preparations are discussed below. However, the treatment of *Anthopleura xanthogramica* or *Anthopleura elegantissima* in this fashion resulted in unsuitable material. *A. xanthogramica* is infected with zooxanthellae which could only be removed by shaking the nematocyst suspensions with ether. Upon centrifugation the nematocysts were found in the sediment and the algal cells along with any remaining fine tissue debris stayed in the ether phase which had a gelatinous consistency. With this modification highly purified suspensions could be obtained, but the nematocysts could not be artificially discharged. While it proved possible to obtain, in protected, darkened areas, specimens of *A. elegantissima* which did not contain symbiotic algae, the nematocysts obtained from these animals were also not susceptible to artificial discharge.

Characteristics of Nematocyst Suspensions from Metridium senile fimbriatum

Approximately 0.5 gram, dry weight, of nematocysts was obtained from 500 grams, wet weight, of this species. The material was all but completely free of tissue debris and sand when examined microscopically. Continued differential centrifugation neither increased the per cent hexosamine content of 3.1–3.2% after hydrolysis nor decreased the total nitrogen content which was 10.2–10.4%. The per cent composition of different batches of nematocysts agreed within experimental error. A dried preparation could be obtained by washing suspensions with a solution of glycerine and distilled water, 1 : 1 by volume, followed by 95% ethanol and ether and drying in a desiccator.

The half-life of a purified nematocyst suspension appears to depend at least partly upon the osmotic pressure exerted by the suspending medium. When kept in the refrigerator the time required for discharge of one half of the nematocysts was 12 hours in 1 *M* sucrose in sea water, 7 days in 1 : 1 glycerine and distilled

water, at least three months in 95% ethanol, and over 6 months for dried material. These observations are in agreement with those of Glaser and Sparrow (1909). The dried material would probably keep indefinitely (Weill, 1926). However, neither alcoholic suspensions nor dried material exhibits any toxicity. Both ether and alcohol effectively detoxify the nematocysts.

The spectrum of nematocyst types, cnidom, for *M. senile fimbriatum* has been described recently by Hand (1955). Table I gives the differential count for each of the types found in the suspensions. Some of the types have been divided into size categories which represent approximate mean dimensions. This variation in size with respect to a particular type makes a physical separation of one type from another extremely difficult. Until it can be determined that the toxin of

TABLE I

Differential count of nematocysts in suspensions from Metridium senile fimbriatum

Nematocyst type	Size (microns)	Counted	Per cent
Microbasic b-mastigophore	60 × 5	15	7.4
	30 × 4	6	2.9
	10 × 3	6	2.9
Microbasic p-mastigophore	20 × 3	2	1.0
	10 × 5	14	6.9
Microbasic amastigophore	70 × 7	10	4.9
	30 × 5	21	10.4
	10 × 4	8	3.9
Basitrich	20 × 4	20	9.8
	13 × 2	16	7.8
Atrich	24–47 × 7–15	2	1.0
Holotrich	13–23 × 4–6	7	3.4
Spirocysts	12–30 × 4	76	37.4
	Totals	203	99.7

one type is the same or different from the toxin of another type, it seems desirable to present such counts as a part of the description of the material whose toxicity is under investigation.

Nematocyst Toxin from Metridium senile fimbriatum

The purified suspensions contain 37–39% discharged nematocysts. The remainder can be artificially discharged by treatment with distilled water, methylene blue, weak acid, weak base, sodium thioglycolate, or sodium taurocholate. In order to obtain the maximum release of toxin, the nematocysts were placed in distilled water for 12 to 18 hours. After such a period all but approximately 1% are discharged. Such "normal" discharge was found to be just as effective a means of obtaining the toxin as grinding and extraction. Discharge released 21.8% of the dry weight of the nematocysts. Grinding with carborundum in a mortar and extraction with distilled water removed 21.7%. Discharge in a test tube may be followed with the naked eye. The tubes everted from the nematocysts become entangled and eventually form a slimy, cottony sediment.

Some information as to the chemical nature of the toxin has been obtained

(Phillips, 1956). Hydroxy-indoles were detected on paper chromatograms. In an attempt at isolation of these substances from large amounts of nematocysts, the content of 5-hydroxy-indoles was followed quantitatively, using the method of Mitoma *et al.* (1956) during the purification of the nematocysts. As the suspension became more and more free of tissue components, the level of 5-hydroxy-indoles dropped steadily. This would suggest that these substances are not a part of the toxin but instead represent a soluble tissue component.

Various marine invertebrates were tested for their susceptibility to extracts of the nematocysts of *Metridium senile fimbriatum*. The animal found to be the most convenient for assay purposes was *Littorina planaxis,* a small snail from the high intertidal zone. This animal normally has no contact with coelenterates of any sort, at least during its post-larval and adult stages. When placed upside down in sea water it rapidly rights itself and moves out of the water to a relatively dry

TABLE II

Per cent inhibition of the righting response of Littorina planaxis by distilled water extracts of the nematocysts of Metridium senile fimbriatum

Dose, micrograms	Time, minutes				Hours				Days			
	5	10	20	40	1	2	4	8	1	2	4	8
150.0	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	80%*
75.0	100	100	100	100	100	100	100	100	80	80	70	40**
37.5	100	100	100	100	100	100	100	100	70	30	30	0
18.75	100	100	100	100	100	90	80	50	0	0	0	0
9.375	100	90	80	80	80	80	40	20	0	0	0	0
7.5	100	100	90	30	20	0	0	0	0	0	0	0
0.75	100	80	40	20	0	0	0	0	0	0	0	0
0.00	70	60	40	0	0	0	0	0	0	0	0	0

* 8/10 dead.
** 4/10 dead.

place. It was found that the time required for this righting and withdrawal from the water could be prolonged by addition of the toxin and the length of inhibition is dependent upon the concentration of the toxin. With very high doses of the toxin the time was infinitely extended since there was a resulting death of the snail.

The titrations of toxicity were carried out as follows: The various doses of toxin were prepared in 1 ml. of sea water and placed in flat bottomed tubes, 25 × 95 mm. Ten tubes of each dose were prepared so that ten snails per dose could be tested simultaneously. The snails were dried with a towel, and the water in the mantle cavities was removed by gentle pressure on their opercula with a towel-covered probe. They were then dropped into the toxin and shaken so that the snails were upside down and the cup of the shell was filled with toxin. One milliliter of the toxin dilution was insufficient to cover the snail, so that the amount of diluted toxin actually involved in the test was the amount which was contained in the cup of the shell and was ultimately drawn into the mantle cavity. The time at which the snails were first dropped into the diluted toxin was noted, and the time at which they righted themselves was noted. Table II shows the results obtained

with various doses of the toxin. The dose is expressed in micrograms dry weight of toxin contained in 1 ml. of sea water. For convenience, 1½ hours was taken as the period of observation in subsequent titrations.

Since *Littorina* is exposed to fresh water in the form of rain at not infrequent intervals, it seemed unlikely that the righting response would be affected by dilution of the sea water with distilled water. However, since the toxin was obtained by discharge of the nematocysts in distilled water, the effect of the dilution of the sea water by the addition of toxin was determined. No effect due to dilution could be found over the range used in the test.

The snails employed in the titrations show a considerable variation in size. On the basis of body weight, including shell, they vary from 1.15 to 0.33 grams. Yet their response to the toxin did not appear to be correlated with body weight. In order to explain this observation a group of snails was weighed independently before and after the removal of the fluid from the mantle cavity. In this way the volume and weight of the effective dose received by the snails of various weights

TABLE III

Per cent inhibition of the righting response of Littorina planaxis by toxin of Metridium senile fimbriatum obtained by discharge of nematocysts in two different media

	Toxin obtained by discharge in:	
Dose (micrograms/ml. of sea water)	Distilled water % Inhibited	Sodium thioglycolate % Inhibited
30.0	90	100
15.0	80	50
7.5	20	40
3.75	10	0
1.875	0	0
ED_{50}	12	11.5
$f_{ED_{50}}$	1.52	1.75
95% confidence limits of ED_{50}	7.9–18.2	6.5–20.4

were determined. It was found that the dose received by each snail is proportional to its body weight. The volume of sea water that contains the toxin and is drawn into the mantle cavity varies from 0.10 to 0.01 ml., depending on the size of the snail. This means that the effective dose of toxin is $\frac{1}{10}$ to $\frac{1}{100}$ of the amount present in the milliliter of toxin dilution.

The toxin was assayed using five doses and ten snails per dose. The titrations were terminated after 1½ hours. Since there was a possibility that the toxin was sensitive to oxygen, toxin obtained by discharge of the nematocysts in 0.05% sodium thioglycolate was compared with toxin obtained by discharge in distilled water. The method of Litchfield and Wilcoxon (1949) was used to obtain: (a) the median effective dose, ED_{50}; and (b) the factor, $f_{ED_{50}}$, for obtaining the 95% confidence limits of the ED_{50}. The curves were found to be parallel within experimental error. Table III shows the results of these titrations and includes the parameters mentioned above. It does not appear that the toxin is particularly oxygen-labile.

SUMMARY

1. A method of obtaining purified suspensions of nematocysts has been developed, and a method of obtaining the toxin they contain has been described.

2. A method of assay, using inhibition of the righting response of *Littorina planaxis*, has been shown to be applicable for toxicity titrations.

3. Further work on the chemical and immunochemical characterization of the toxin is planned, as well as the use of the toxin for studies of antitoxic immunity in marine invertebrates.

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